

Improving plant genetic engineering by manipulating the host

Stanton B. Gelvin

Dept of Biological Sciences, Purdue University, West Lafayette, IN 47907-1392, USA

***Agrobacterium*-mediated transformation is a major technique for the genetic engineering of plants. However, there are many economically important crop and tree species that remain highly recalcitrant to *Agrobacterium* infection. Although attempts have been made to 'improve' transformation by altering the bacterium, future successes might come from manipulation of the plant. Recent studies that identified several plant genes involved in *Agrobacterium*-mediated transformation, and their over-expression in currently transformable species, suggest that this approach holds great promise for improving the transformation of recalcitrant, but agronomically important, crops.**

The genetic transformation of plants by the Gram-negative soil bacterium *Agrobacterium tumefaciens* is the only known example of natural inter-kingdom genetic exchange. During the course of infection, *Agrobacterium* processes a region of DNA, the T-(transferred) DNA, from a resident Ti-(tumor inducing) plasmid and transfers T-DNA and several virulence (Vir) proteins to plant cells. These Vir proteins include VirD2 (an endonuclease that is involved in T-DNA processing and that is covalently attached to the 5' end of the processed single-stranded T-strand), VirE2 (a sequence-independent single-stranded DNA binding protein that probably coats T-strands) and VirF. It has been proposed that VirD2, VirE2 and the T-strand form a complex, the T-complex, which is the probable molecular form that is transported through the plant cytoplasm into the nucleus [1]. The site of assembly of this complex (within the bacterium or within the plant cell) remains somewhat controversial (reviewed in [2]).

In nature, the result of this genetic transformation process is the generation of crown gall tumors on infected plant tissues. However, scientists can delete T-DNA-encoded oncogenes and replace them with genes of agronomic value that are subsequently transferred to and expressed within plant cells in a procedure known as *Agrobacterium*-mediated genetic transformation. This process is considered preferable to genetic transformation by artificial approaches (e.g., electroporation, microinjection, or 'biolistic' bombardment of cells with highly accelerated naked DNA molecules) because of the ease and low cost of the procedure and because of the relatively low complexity of intact transgenes integrated into the plant genome. Thus, *Agrobacterium*-mediated transformation has been used

not only to generate a wide variety of transgenic plants [3] but also for the genetic transformation of fungi [4–6] and even human cells [7].

Although *Agrobacterium*-mediated transformation would appear to be the method of choice for plant genetic engineering, many economically important plant species, and elite cultivars of particular species, are highly recalcitrant to the method. In particular, the efficient genetic transformation of soybeans, cotton, cereal grains, many legumes and tree species of horticultural and industrial importance remains problematic. Scientists have attempted to increase the efficiency of plant transformation by identifying or manipulating more highly virulent *Agrobacterium* strains (e.g., [8]) or by improving plant culture conditions [9]. Although the use of 'super-virulent' strains and 'super-binary vectors' containing additional copies of various *vir* genes have increased the transformation of many plants, including cereals, limits might have been reached in these efforts [2]. An alternative approach to increasing plant transformation might lie in manipulation of the plant itself. To accomplish this, it is first necessary to identify plant genes and their respective encoded proteins that are involved in the transformation process. Several publications within the past two years suggest that this approach could be fruitful.

Plant genes involved in *Agrobacterium*-mediated transformation: histones and T-DNA integration

Several years ago my laboratory embarked on a series of experiments to identify plant genes involved in *Agrobacterium*-mediated transformation using a classical genetic approach. We developed a screening protocol [10] to identify mutants that were 'resistant to *Agrobacterium* transformation' (*rat* mutants) in a population of T-DNA-mutagenized *Arabidopsis* plants [11]. To date, we have screened > 16 000 mutagenized plants and identified (using forward and reverse genetic methods) > 100 *rat* mutants. Of particular interest is *rat5*, a mutant containing a T-DNA insertion in the 3' untranslated region of the histone H2A-1 (*HTA1*) gene [12]. *rat5* mutant plants could be transiently but not stably transformed by *Agrobacterium*, suggesting that the block to transformation resides at the T-DNA integration step (T-DNA molecules do not necessarily need to integrate into the plant genome to express transiently [13,14]). Over-expression of wild-type copies of the *HTA1* gene in *rat5* mutant plants could restore transformation proficiency. More importantly, over-expression of the *HTA1* gene in wild-type *Arabidopsis* plants significantly increased

Corresponding author: Stanton B. Gelvin (gelvin@bilbo.bio.purdue.edu).

susceptibility to *Agrobacterium*-mediated root transformation [12] and slightly increased the transformation frequency by the 'flower dip' method, initially suggesting that histone H2A expression, and hence T-DNA integration, might be limiting in the transformation of target cells (Fig. 1a). Yi *et al.* [15] recently demonstrated that *Arabidopsis* elongation zone root cells that express the *HTA1* gene are most highly susceptible to *Agrobacterium*-mediated transformation. Alteration of the expression

pattern of *HTA1* in roots by wounding, phytohormone treatment or *Agrobacterium* infection changed the pattern of transformation susceptibility in a corresponding manner, suggesting that *HTA1* expression might serve as a molecular 'marker' for predicting cells most prone to *Agrobacterium* infection.

Improving nuclear transport of the T-complex

Another approach has been used by Vitaly Citovsky's laboratory to identify plant proteins involved in the *Agrobacterium* transformation process. Using a yeast two-hybrid interaction approach, Ballas and Citovsky [16] identified an importin- α (AtKap α) that interacted specifically with a nuclear localization signal (NLS) sequence within VirD2. Nuclear localization of the T-complex is an essential step in *Agrobacterium*-mediated transformation, and both VirD2 and VirE2 contain NLS sequences. Mutation of the VirD2 NLS impairs T-complex nuclear translocation and transformation to various extents [12,17,18]. However, Citovsky's group was not able to show, using a yeast two-hybrid system, interaction of VirE2 with AtKap α [16,19]. Rather, they showed that VirE2 interacted with the *Arabidopsis* protein VIP1 [20]. VIP1 could mediate VirE2 nuclear translocation and tobacco plants expressing an anti-sense construction of the *VIP1* gene were recalcitrant to subsequent *Agrobacterium* transformation [20].

Recently, Tzfira *et al.* [19] demonstrated that over-expression of VIP1 in transgenic tobacco plants could substantially increase the efficiency of *Agrobacterium*-mediated transformation (Fig. 1b). Not only were VIP1 transgenic plants more susceptible to stable transformation (as evidenced by an increase in efficiency in generating hygromycin-resistant shoots) but they were additionally more receptive to transient transformation (as evidenced by an increase in transient GUS activity expressed from a T-DNA-encoded *gusA*-intron gene). VIP1 transgenic plants also expressed processed mRNA from a T-DNA-encoded *gusA*-intron gene more quickly after infection than did wild-type plants (4 hr compared with 12 hr), suggesting more rapid and/or efficient T-strand nuclear targeting. Thus, over-expression of a gene involved in the nuclear targeting step of *Agrobacterium*-mediated transformation, as well as genes such as *HTA1* involved in the T-DNA integration process, can potentially increase the transformation efficiency of plants.

Future prospects

Various steps in the *Agrobacterium*-mediated transformation of plant cells are indicated in Figure 2. Events occurring within the bacterium, including signal perception, *vir* gene induction, T-strand processing, and T-DNA/Vir protein transport, are reasonably well understood, although the formation and functioning of the Type IV secretion apparatus necessary for T-DNA and Vir protein export remain an active area of research. We currently understand considerably less about events that take place on or within the plant cell, including bacterial attachment, T-DNA and Vir protein transfer, cytoplasmic trafficking, nuclear targeting, and T-DNA integration. Each of these steps might be considered a target for

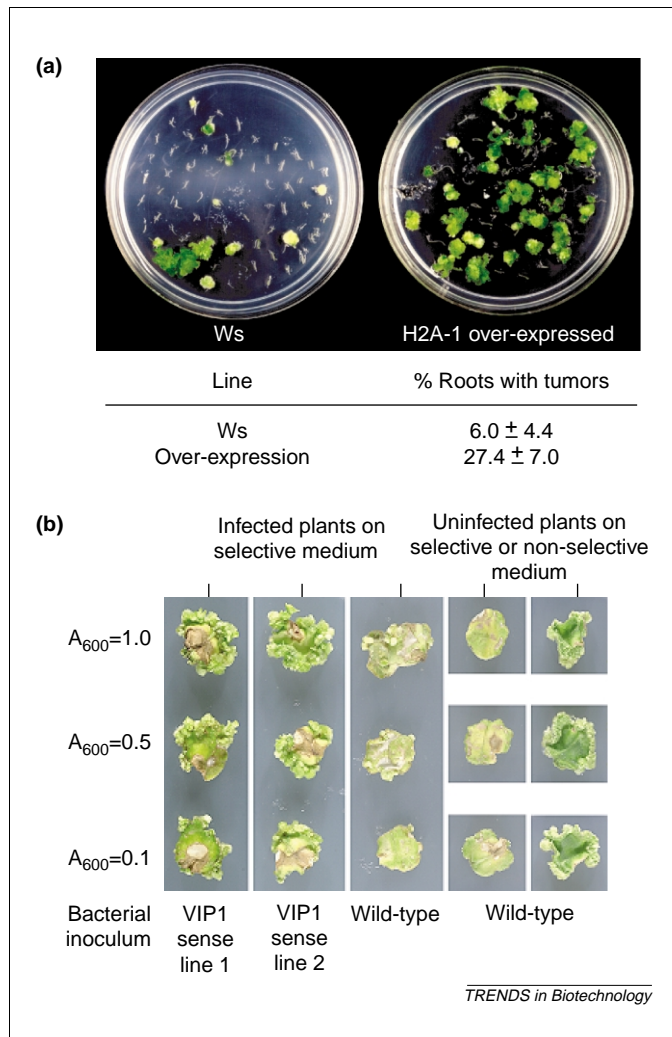


Fig. 1. (a) Histone *H2A-1* cDNA over-expression in wild-type plants results in increased susceptibility to *Agrobacterium* transformation. Transgenic *Arabidopsis* plants expressing a histone *H2A-1* cDNA under control of a CaMV 35 S promoter were generated and root segments of these plants and wild-type (ecotype Ws) plants were infected with a dilute culture of the oncogenic *A. tumefaciens* strain A208. After 2 days, the root segments were transferred to media containing antibiotics (to kill *Agrobacterium*) and lacking phytohormones (to select for tumors). The plates were photographed one month later. Note the fourfold to fivefold increased frequency of tumorigenesis on roots of plants over-expressing the *HTA-1* gene, compared with wild-type Ws control plants, as quantified in the table summarizing the results of >10 independent transgenic lines. (b) Over-expression of VIP1 results in increased transformation of transgenic tobacco plants. Transgenic tobacco plants were generated that express the VIP1 open reading frame under the control of a CaMV 35S promoter. Leaf disks from two lines of transgenic plants, and a wild-type control plant, were infected with various concentrations of a disarmed *Agrobacterium* strain containing a binary vector encoding a plant-expressible *hptII* gene, and disks were placed on hygromycin-containing shoot regeneration medium. Note that more shoots were produced on selective medium from VIP1 transgenic plants than from wild-type plants (left portion of figure), and that wild-type plants regenerated shoots normally when placed on non-selective medium but not on selective medium (right portion of the figure). Adapted, with permission, from [19].

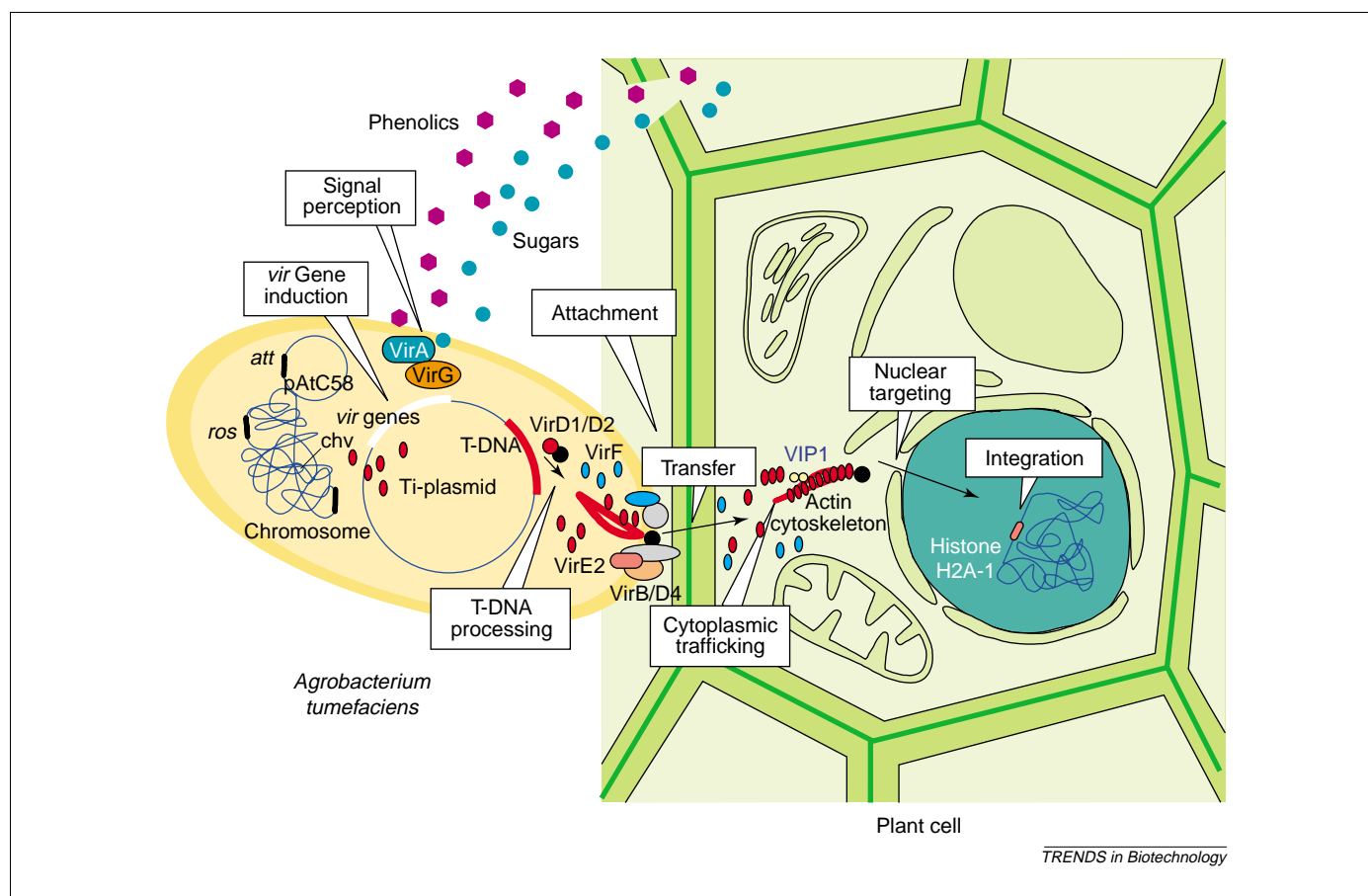


Fig. 2. Model for the process of *Agrobacterium*-mediated transformation. This model depicts the various steps in the transformation process, including those occurring within the bacterium (perception of phenolic and sugar signals from wounded plant cells, virulence gene induction, T-strand processing from the Ti-plasmid and the subsequent export of the processed T-strand and Virulence proteins) and those involving the plant cell (bacterial attachment, T-strand and Virulence protein transfer, cytoplasmic trafficking and nuclear targeting of the T-complex, and T-DNA integration into the plant genome). VIP1 is hypothesized to influence events in the plant cytoplasm, whereas histone H2A-1 influences events within the nucleus.

manipulation. Considering the recent successes in over-expression of several plant genes involved in transformation, what can we expect in the future? Genetic and protein interaction approaches mentioned above, combined with genomic methods (Veena, H. Jiang, R.W. Doerge, and S.B. Gelvin, unpublished observations), have identified >100 *Arabidopsis* genes involved in *Agrobacterium*-mediated transformation. These genes encode proteins involved in bacterial attachment (an arabinogalactan protein), T-DNA transfer (a xylan synthase), nuclear targeting (importins and actins, perhaps indicating the use of the actin cytoskeleton as a 'track'; P. Rao and S.B. Gelvin, unpublished observations), and T-DNA integration (histones and chromatin modifying proteins). It seems likely that some of these genes could serve as targets for genetic manipulation to increase transformation. However, there are probably many surprises in store for us. For example, although mutation of the histone H2A-1 gene *HTA1* results in decreased T-DNA integration, over-expression of *HTA1* might affect transformation by another mechanism. Recent results from my laboratory indicate that over-expression of *HTA1* in many different *rat* mutant lines can restore transformation-susceptibility to these otherwise highly recalcitrant mutants (L.-Y. Lee, S. Davis, X. Sui, and S.B. Gelvin; unpublished observations). Many of these mutations are in

genes not known to be involved in T-DNA integration. Thus, *HTA1* over-expression could generally 'sensitize' plant cells to *Agrobacterium*-mediated transformation in an as yet unknown way. Detailed investigation of plant gene function and expression patterns in the future will probably lead to novel methods for improving the genetic engineering of plant cells.

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Molecular computing revisited: a Moore's Law?

Michael S. Livstone, Danny van Noort and Laura F. Landweber

Dept of Ecology and Evolutionary Biology, Princeton University, Princeton, NJ 08544, USA

Moore's Law states that the processing power of microchips doubles every one to two years. This observation might apply to the nascent field of molecular computing, in which biomolecules carry out logical operations. Incorporation of new technologies that improve sensitivity and throughput has increased the complexity of problems that can be addressed. It is an ultimate goal for molecular computers to use the full potential of massive parallelism.

In 1994, Leonard Adleman performed the first successful experiment that solved a mathematical problem using DNA [1]. In doing so, he provided a concrete demonstration of what many biologists had long believed: that the components that encode the building blocks of life are, at their core, a complex computer that can process information in many different ways. Since then, the field of 'molecular computing' including DNA, RNA and other information-bearing biopolymers, has been the subject of numerous scientific articles, books, international conferences and even a posthumously published novel by Robert Ludlum [2]. Two factors drive progress in molecular computing: the use of the inherent massive parallelism of nucleic acid interactions to perform many computations simultaneously and the incorporation of new technologies into nascent 'computers' to improve automation and sensitivity. Here, we examine the nature of this parallelism and the technologies that have been applied to molecular computing (see Box 1).

Representative molecular computing problems

Important considerations in molecular computation include the methods of encoding information, generating potential solutions and selecting and identifying correct solutions. As a framework, we discuss two NP-complete problems that have been approached by researchers, Hamiltonian path (HP) and Satisfiability (SAT).

Adleman's original problem was an instance of HP, which asks: 'Given a set of cities, a starting point, an ending point, and the (one-way) routes connecting them, is it possible to visit every city exactly once?' One algorithm generates all possible orderings of cities. The difficulty increases exponentially with the number of cities, so that even with only seven, there are potentially 120 ways to traverse the five intermediates. Adleman's approach [1] (reviewed in [5]) was to encode the cities as unique 20-nucleotide (nt) sequences of single-stranded DNA and the paths between them as 20-nt sequences complementary to half the sequence of each of the cities they connect. He generated all potential solutions by combining the sequences representing cities and routes in a test tube and isolated the unique correct solution using a three-step process. First, routes that begin and end in the correct cities were selected by PCR with specific primers. Next, routes of the correct length, 140 base pairs, were gel-isolated. Finally, he sequentially purified those molecules that hybridized with each of the five intervening 'cities'. Including verification, the process took approximately one week of bench time.

The most difficult problem solved on a molecular computer to date [6] is a 20-variable, 24-clause instance

Corresponding author: Laura F. Landweber (lfl@princeton.edu).